BIOORGANIC CHEMISTRY 15, 194–211 (1987)

REVIEW

Quinone Methide Sclerotization: A Revised Mechanism for β-Sclerotization of Insect Cuticle

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Received September 17, 1986

Molecular mechanisms responsible for the stiffening and tanning of insect cuticle are reviewed. Two mechanisms, viz., quinone tanning and β-sclerotization, both involving catecholamine derivatives as sclerotizing precursors, are known to strengthen the cuticle. Quinone tanning mechanism invokes the generation and reactions of *o*-benzoquinones as the sclerotizing agents, whereas β-sclerotization dictates the activation of catecholamine side chains prior to their incorporation into cuticle. The reactive intermediate for the latter process was proposed by other workers to be 1,2-dehydro-*N*-acetyldopamine and its quinone. The role of these two compounds in β-sclerotization is critically evaluated. Based on our observation that incubation of cuticular enzyme from *Sarcophaga bullata* with 4-alkylcatechols results in the production of soluble side chain oxygenated compounds and the formation of catechol–cuticle adducts, an alternate mechanism for β-sclerotization is proposed. This mechanism calls for the generation of quinone methides, tautomers of 4-alkylquinones, as the initial products of oxidation of catecholamine derivatives in cuticle. Quinone methides formed spontaneously react with available nucleophiles in cuticle, resulting in the generation of catechol–cuticle adducts and side chain hydroxylated products. Further oxidation of adducts and coupling to other structural units ensure crosslinking of cuticular components. The proposed quinone methide sclerotization accounts for all the chemical observations made on the β-sclerotized cuticle.

The greatest success of insects as terrestrial animals is in part due to their tough exoskeleton which provides protection against desiccation, forms a formidable barrier for invading microorganisms and other parasites, allows the growth and development of insect, serves as an anchorage for various muscles, and is responsible for several other functions (*1, 2*). The exoskeleton of the insect, also known as cuticle, is a complex architecture consisting mainly of chitin, protein, and catecholamine derivatives. Insect cuticle does not expand readily and continuously; as a result, insects must often shed their old cuticle and make a new cuticle to allow for growth. The newly formed cuticle is usually soft and pale, and soon it becomes hard and tanned by a process known as sclerotization. During larval–pupal transformation, sclerotization aids the hardening of the pupal cuticle and affords protection for the pupae. Similar hardening reactions strengthen the egg cases of several insects. Sclerotization of exoskeleton is essential to the survival

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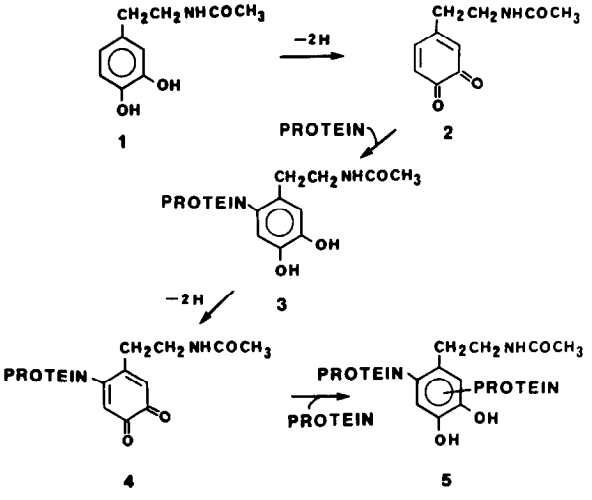
of insects; without it the organism would be vulnerable to its environmental enemies and die from desiccation. Thus, arrest of sclerotization can lead to the death of insects and hence this process lends itself as an attractive target for the development of insecticides. A clear understanding of molecular mechanisms underlying this process will enable us to achieve this goal. Unfortunately, despite the availability of extensive and diverse literature concerning sclerotization, studies at the mechanistic level are few and far between [for reviews, see (*2, 3*)]. Entomologists have been satisfied with studies on the ultrastructural details of the cuticle, while biochemists have limited their studies to the biochemical aspects of cuticle synthesis at the superficial level. The purpose of this article is to draw the attention of organic chemists to an interesting area in insect biochemistry, where chemical mechanisms play a major role in strengthening the cuticle. Basic study of these mechanisms can eventually lead to the modeling of appropriate inhibitors which might become ultimately new types of insecticides.

The formation and deposition of hardened regions in the exoskeleton require precise integration of several synthetic and catabolic pathways in insects. The bulk of the carbon skeleton is either directly transferred to the integument or consumed in reactions aiding the synthesis of cuticle. Sclerotization of cuticle follows its synthesis. Coincident with sclerotization are (a) the mobilization of catecholamine derivatives to cuticle; (b) the decrease in solubility of structural proteins and their increase in resistance to enzymatic and chemical degradation; (c) extrusion of water from cuticle; (d) reorientation of chitin fibrils; (e) formation of chitin–protein, as well as protein–protein, crosslinks; and (f) pigmentation. The joining of two or more structural units by the introduction of a covalent crosslink calls for the reactions of structural protomers with bifunctional reagents known as sclerotizing agents.

As early as 1940, Pryor characterized quinones to be one type of sclerotizing agent (*4, 5*). His studies with the hardening and darkening of cockroach ootheca (egg capsules), which is devoid of chitin, suggested that 3,4-dihydroxybenzoic acid, a catecholamine derivative, is oxidized to its corresponding quinone derivatives by cuticular phenoloxidase and is incorporated into the structural proteins of the egg capsule (*4*). Subsequently, results from various laboratories strengthened this quinone tanning hypothesis (*6–10*). The quinone tanning hypothesis in its simplest form is presented in Scheme 1. According to this hypothesis, catecholamine derivatives such as *N*-acetyldopamine, **1,** or *N*-β-alanyldopamine, which are characterized as precursors for sclerotizing agents (*11, 12*), are secreted by epidermal cells into the cuticle where they come into contact with cuticular phenoloxidase. The quinones, **2,** formed by the interaction of cuticular phenoloxidase with sclerotizing precursors, then react with available nucleophiles on structural proteins, yielding catechol–protein adducts, **3.** The adducts, upon further oxidation and coupling with another protein, yield protein–protein dimers, **5.** The arylated proteins rapidly undergo polymerization by crosslinking at various loci. These addition reactions enable the cuticular proteins to compress sufficiently to extrude water from cuticle and decrease the solubility and susceptibility to proteolysis of all the cuticular proteins.

The ε-amino group of lysine, the N-terminal amino groups, the imidazole group of histidine, the thiol group of cysteine, and the thioether group of methionine are

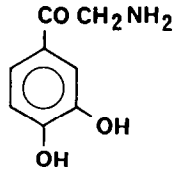


SCHEME 1

potential sites for such crosslinking in proteins (*10*). Although the quinone tanning hypothesis has been widely accepted for nearly four decades, several workers have recently doubted the occurrence of such reactions. Hackman and Goldberg (*13*), who carried out amino acid analyses of cuticle before and after tanning, found very little difference in lysine content. They suggested that probably quinone undergoes polymerization to a melanin-like polymer to which structural proteins adhere very tightly by hydrophobic interactions and hydrogen bonding. The hydrophobic hypothesis was also endorsed by Vincent and Hillerton (*14, 15*) on the basis of mechanical properties of cuticle. In addition, Peter (*16*), who studied the *in vitro* oxidation of catecholamine derivatives in the presence of β-alanine, failed to observe the formation of any Michael-1,4-addition products between quinones and β-alanine.

To test the validity of the quinone tanning hypothesis, we examined the total acid hydrolysates of the cuticles previously radiolabeled with aromatic precursors and amino acids. Dowex column chromatography of cuticular hydrolysates revealed that histidine and lysine were converted to aryl conjugates, clearly indicating the operation of the quinone tanning hypothesis (*17–20*). Recently, we conducted some *in vitro* model sclerotization studies with *N*-acetyldopamine, mushroom tyrosinase, and lysozyme (test protein) and observed not only dimer and trimer formation but also oligomerization and precipitation of the protein in the reaction mixture (M. Sugumaran, unpublished results). These studies further ascertain the quinone tanning hypothesis for sclerotization.

An alternate mechanism for the tanning reaction was found to operate in several cuticles, and the rest of this paper will deal with the sclerotizing agent participating in this mechanism. Unlike the quinone tanning mechanism where the aromatic ring provides the loci for protein attachment, the alternate mechanism, named β-sclerotization, seems to use the side chain of the catecholamine derivative for crosslinking the structural protomers. In 1970, Andersen isolated 2-amino-3′,4′-dihydroxyacetophenone, **6**, from the hydrolysates of sclerotized cuticle (*21*). To



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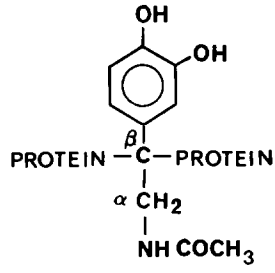

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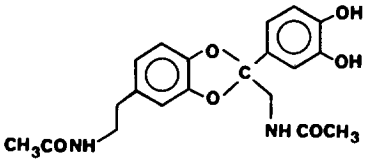
account for the generation of **6,** he suggested (*22*) that sclerotizing precursors such as *N*-acetyldopamine are somehow activated and incorporated into cuticle such that upon hydrolysis they release **6** and related compounds. The putative crosslink was proposed to be **7.** Since this mode of crosslinking involves protein attachment to the β-carbon atom of *N*-acetyldopamine, it was termed as β-sclerotization. In support of this mechanism, he also observed the release of tritium from the side chain of tritiated *N*-acetyldopamine during incubation with cuticular enzymes (*23*). Furthermore, proteolytic digests of cuticle generated soluble peptides to which catecholamines were bound covalently. These bound catechols initially did not show any spectral characteristics corresponding to **6.** However, upon acid hydrolysis, the carbonyl group was freed and **6** was liberated from the peptides, indicating the presence of β-crosslinks (*21*).

Subsequently, Andersen and other workers isolated a variety of catecholamine derivatives from the cuticular hydrolysates of a number of organisms, confirming the widespread occurrence of β-sclerotization (*24–29*). These compounds can be grouped into three classes of compounds as shown in Table 1. Group I catechol derivatives, as mentioned earlier, are precursors for sclerotizing agents, while group II and group III catechols seem to arise from β-sclerotized tissues by hydrolysis and are useful in structural elucidation of the sclerotizing agent. Of these compounds, **12** and **6** arise as the hydrolysis products of **11** and **14,** respectively, while **16, 17, 19,** and **20** are probable degradative products of **15** (*24*). Compounds **13** and **18** are limited for very few cuticles and are of questionable origin. Therefore, they need not be considered for mechanistic purposes. This leaves compounds **11, 14,** and **15** as the key intermediates in determining the structure of the β-sclerotizing agent.

During the course of investigation, Andersen (*30*) isolated a dimer of *N*-acetyldopamine by incubating **1** with cuticular enzymes. Hydrolysis of this compound with 1 N HCl at 100°C for 3 h yielded mainly equimolar amounts of **8** and **15,** while hydrolysis with 6 N HCl for 30 min at 100°C gave **1** with a mixture of **6** and **14.** On methylation with diazomethane, the dimer yielded a dimethoxy derivative which on acid hydrolysis gave **8** and 2-hydroxyl-3′,4′-dimethoxyacetophenone. Based on these studies, structure **21** was proposed for the dimer (*30*).

Although **21** accounts for some of the hydrolysis products and tritium release (*23*), it does not account for the nearly quantitative recovery of 2-hydroxyl-3′,4′-dihydroxyacetophenone, **15,** on acid hydrolysis of the dimer. Since the dimer



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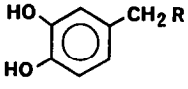
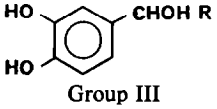
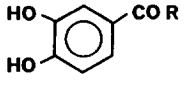
seems to be formed during the natural hardening of the cuticle, solving its structure can lead to the elucidation of the structure of the reactive intermediate for β-sclerotization. Therefore, Andersen's group performed a detailed investigation on this compound. Following ^{1}H and ^{13}C NMR and mass spectral studies (*31–33*), the structure of this dimer was revised to **22.** It was also reported that the dihydroxyphenyl-substituted benzodioxan derivative upon acid hydrolysis produced the monomeric catechols **1** and **15** (Scheme 2).

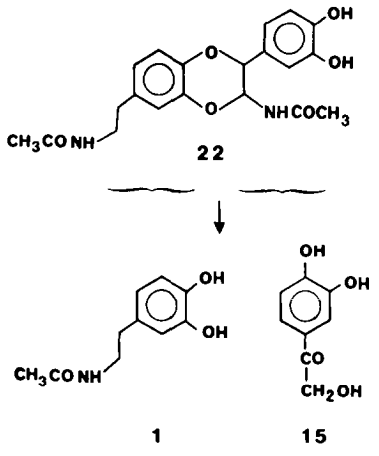
In consideration of the dimer formation, a scheme was proposed in which the sclerotizing precursor, *N*-acetyldopamine, was activated at both α and β positions before incorporation into cuticle (Scheme 3). The activated species was later suggested to be 1,2-dehydro-N-acetyldopamine, **25** (*31, 32*). Subsequently, **25** was isolated from sclerotized cuticle by treatment of the cuticle with boiling 1 N sodium hydroxide for a couple of minutes (*34*). Scheme 4 was proposed by Andersen (*34, 35*) to explain the β-sclerotization. According to this scheme, the widely used sclerotizing precursor, **1,** is converted to **25** by a yet uncharacterized desat-

TABLE 1

STRUCTURES OF CATECHOLIC COMPOUNDS ISOLATED FROM CUTICLE[a]

Structure	No.	Name	R
Group I 	**1**	*N*-Acetyldopamine	$-CH_2NHCOCH_3$
	8	Dopamine	$-CH_2NH_2$
	9	3,4-Dihydroxyphenethyl alcohol	$-CH_2OH$
	10	3,4-Dihydroxyphenylacetic acid	$-COOH$
Group II 	**11**	*N*-Acetylnorepinephrine	$-CH_2NHCOCH_3$
	12	Norepinephrine	$-CH_2NH_2$
	13	3,4-Dihydroxyphenylglycol	$-CH_2OH$
Group III	**6**	2-Amino-3′,4′-dihydroxyacetophenone	$-CH_2NH_2$
	14	*N*-Acetyl-2-amino-3′,4′-dihydroxyacetophenone	$-CH_2NHCOCH_3$
	**15**	2-Hydroxy-3′,4′-dihydroxyacetophenone	$-CH_2OH$
	16	3,4-Dihydroxyphenylglyoxal	$-CHO$
	17	3,4-Dihydroxyphenylglyoxylic acid	$-COOH$
	18	2-Acetyloxy-3′,4′-dihydroxyacetophenone	$-CH_2OCOCH_3$
	19	3,4-Dihydroxybenzoic acid	$-OH$
	20	3,4-Dihydroxybenzaldehyde	$-H$

Source. References (*24–29*).

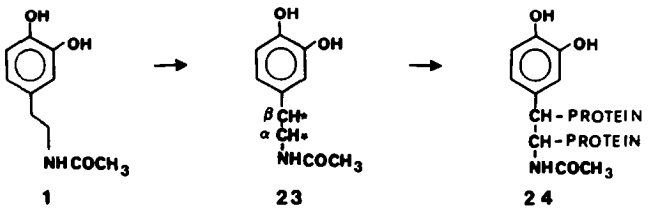


SCHEME 2

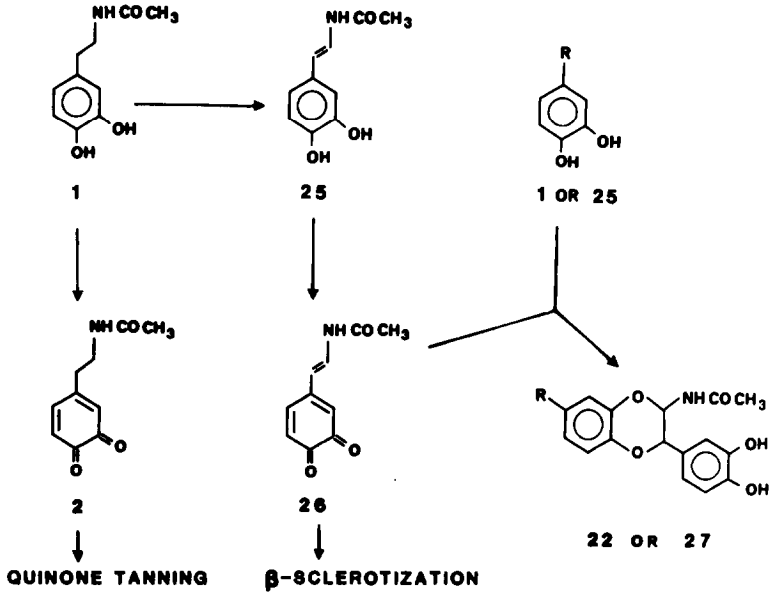
urase. The dehydro compound thus formed is oxidized by cuticular phenoloxidase to its corresponding dehydroquinone, **26,** which is proposed to be the central reactive intermediate for β-sclerotization. The high chemical reactivity of this compound was assumed to manifest itself through its side chain. Thus, its reaction with cuticular components would generate covalently bound catecholic structures in cuticle. Its condensation with either **1** or **25** would supposedly produce **22** or **27,** respectively.

Superficially, it appears that Scheme 4 accounts for most of the observed reactions very well, but a careful analysis reveals that this scheme has several drawbacks. The validity of the above scheme depends heavily on the reactions of two compounds, viz., 1,2-dehydro-*N*-acetyldopamine, **25,** and its quinone, **26.** Chemical considerations, however, speak against their central role in β-sclerotization.

1. This proposal is based on the presence of a desaturase activity in the cuticle. If such a desaturase exists, it might resemble other enzyme systems, such as succinate dehydrogenase or fatty acid dehydrogenase which introduce double bonds in aliphatic chains and hence require oxidized electron acceptors such as NAD, NADP, or FAD as cofactors. However, during catechol oxidation, no such requirements have been found.



SCHEME 3



SCHEME 4

2. β-Sclerotization is ascribed to the reactions of dehydroquinone with cuticular components according to this scheme. But the scheme does not elaborate on the molecular mechanism of adduct formation. While saturated quinone gives dark brown-colored cuticle, it is hard to believe the corresponding unsaturated quinone produces light-colored cuticle. Reactions of this quinone with cuticle are assumed to provide colorless adducts in agreement with the light color of β-tanned cuticle. Quinones with conjugated double bonds absorb light more strongly at higher wavelength regions than their corresponding saturated counterparts. Therefore, they are expected to give colored products rather than colorless adducts.

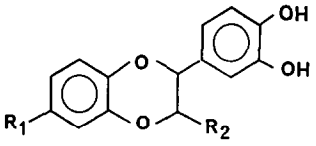
3. It is proposed that the dehydroquinone is highly reactive and reacts through its side chain. Similar quinones with conjugated double bond systems are known in the literature, but they react typically like other quinones, undergoing Michael-1,4-addition reactions on the ring rather than side chain. For instance, chlorogenic acid quinone reacts with amino acids, forming 1,4-addition products rather than side chain adducts (*36, 37*).

4. Cuticular phenoloxidases can oxidize not only *N*-acetyldopamine and *N*-β-alanyldopamine but a wide variety of compounds having catecholic structure and simple side chains at the 4-position. Two of the compounds, viz., 4-methylcatechol and 3,4-dihydroxyphenylacetic acid, **10,** are converted to side chain modified compounds (*38, 39*). The former lacks the β-carbon and the latter has a carboxyl group attached to the α-carbon. Therefore, neither of these compounds can form a double bond at the side chain. Thus any mechanism involving dehydro compound cannot account for the oxidation of these two substrates.

5. The above mechanism is inconsistent with the liberation of compounds hav-

TABLE 2

STRUCTURE OF DIMERS ISOLATED FROM CUTICLE



No.	Substrate	Product	
		R_1	R_2
22	*N*-Acetyldopamine (**1**)	$CH_2CH_2NHCOCH_3$	$NHCOCH_3$
28	**1** + *N*-acetylnorepinephrine (**11**)	$CHOHCH_2NHCOCH_3$	$NHCOCH_3$
29	**1** + 3,4-Dihydroxyphenylglycol (**13**)	$CHOHCH_2OH$	$NHCOCH_3$
30	**1** + 2-Hydroxy-3′,4′-dihydroxyacetophenone (**15**)	$COCH_2OH$	$NHCOCH_3$
31	**1** + 3,4-Dihydroxybenzaldehyde (**20**)	CHO	$NHCOCH_3$
32	**1** + 3,4-Dihydroxyphenethyl alcohol (**9**)	CH_2CH_2OH	$NHCOCH_3$
33	3,4-Dihydroxyphenethyl alcohol (**9**)	CH_2CH_2OH	OH

Note. The hydrolysis products of the R_1 substituted aromatic ring part are the same as their corresponding substrates. The hydrolysis product of the R_2 substituted aromatic ring part is 2-hydroxy-3,4-dihydroxyacetophenone in all cases.

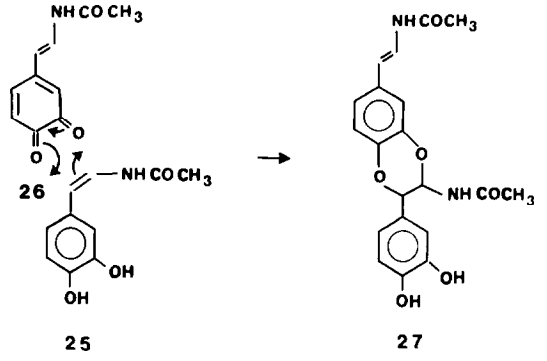
Source. References (*32, 33*).

ing their side chain modified only at the β-position, viz., **11, 14,** and **15,** from cuticular digestion. Since these compounds have their α-methylene group intact, the mechanism involving the dehydro compound is unlikely to account for their formation during hydrolysis.

6. While studying the mechanism of β-sclerotization, Andersen demonstrated with the use of specifically tritiated **1** that only tritium from the β-carbon atom was lost during the incorporation of labeled **1** into the locust cuticle and that tritium located both on the α-carbon atom and on the aromatic ring was retained (*40, 41*). On the contrary, the mechanism involving dehydro compound formation demands the release of tritium from both α and β positions of **1** and hence contradicts the above finding.

7. Incubation of *N*-acetyldopamine with cuticular enzymes from *Sarcophaga bullata* produces *N*-acetylnorepinephrine, **11,** as a product (*39*). This reaction has also been reported for *Manduca sexta* cuticular enzyme (*16*). Compound **11,** thus formed, is shown to be a racemic mixture (*42*). Compound **26** is unlikely to account for the formation of **11.** If a side chain hydrase is found in cuticle, **25** could account for the generation of **11.** However, such an enzyme-catalyzed hydration is unlikely to produce a racemic mixture.

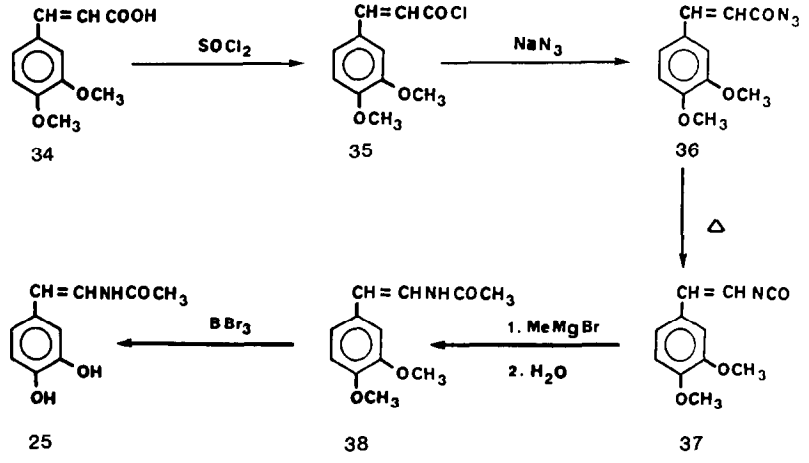
8. The role of dehydroquinone in dimer formation is also questionable. Following the characterization of *N*-acetyldopamine dimer, Andersen and Roepstorff isolated several heterodimers (*32, 33*). The tentative structures of these dimers are given in Table 2. Any mechanism proposed must account for the formation of these compounds also. Although the dehydroquinone, **26,** can react with 1,2-



SCHEME 5

dehydro-*N*-acetyldopamine, **25,** by a Deils–Alder-type reaction as shown in Scheme 5, it cannot participate in such a reaction with other catechols lacking the double bond. These authors suggested that any *o*-diphenols can react with the dehydroquinone to produce the dimer. However, there is little evidence for this proposal. For instance, caffeic acid quinone, which has a double bond, does not react with parent catechol to give the benzodioxan derivative. On the other hand, reaction of **25** with the quinones of different catechols can yield the dimers listed in Table 2 via Diels–Alder addition, but this calls for the oxidation of various catechols to their corresponding quinones and not their side chain activated products. Moreover, catechols with carbonyl side chains such as **20** and **15** are poor substrates for quinone production by phenoloxidases (*43*). Therefore their generation and participation in dimerization are doubtful.

9. Since **25** is a stable compound and an independent chemical synthesis of this compound was not available, we undertook such a study. Using the reactions outlined in Scheme 6, we synthesized this compound and tested its participation in



SCHEME 6

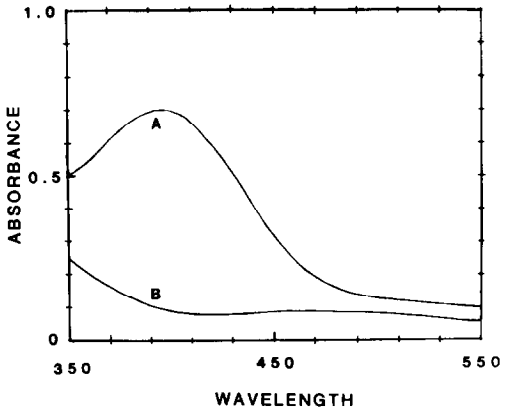


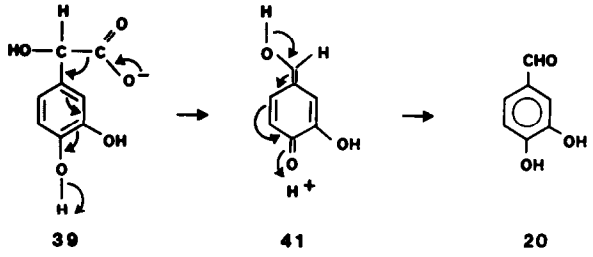
FIG. 1. Visible spectral changes associated with the oxidation of (A) *N*-acetyldopamine and (B) 1,2-dehydro-*N*-acetyldopamine by mushroom tyrosinase. A reaction mixture containing 1 mM substrate in 1 ml of 0.05 M sodium phosphate buffer, pH 6.0, was incubated with 18 μg of mushroom tyrosinase at room temperature for 5 min, and the visible spectral changes associated with the oxidation were recorded against a blank reaction mixture lacking the enzyme. Similar results were obtained with *Sarcophaga bullata* cuticular enzyme also.

β-tanning of sarcophagid cuticle. If this compound is found freely as an intermediate, exogenous addition of this compound and arrest of reaction to a mixture containing radioactive *N*-acetyldopamine and cuticular enzymes will result in trapping of any radioactive dehydro compound formed by the cold compound. Reisolation and radioactive analysis will then reveal its intermediary formation if it is found radioactive. Contrary to this expectation, such a radioactive trapping experiment failed to trap any radioactive dehydro compound, indicating that this compound is not freely formed in the cuticle as an intermediate (*44*).

10. Oxidation of **25** by cuticular phenoloxidase is presumed to produce the corresponding dehydroquinone, **26.** However, in reality its oxidation by either mushroom tyrosinase or cuticular phenoloxidase does not produce **26,** although under the same conditions, **1** is converted to **2** (Fig. 1). Since dehydroquinone is not formed during the enzyme-catalyzed oxidation, its participation in β-sclerotization is also unlikely.

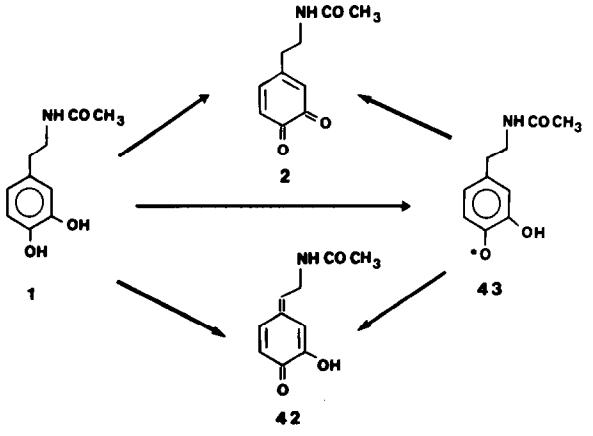
From the foregoing discussion, it is clear that the proposed mechanism for β-sclerotization has serious drawbacks and a revised mechanism is necessary to account for various observations made on β-sclerotized cuticle.

A close examination of the properties of phenoloxidase suggests that an alternate mechanism is possible by invoking the generation of quinone methides in cuticle. Mushroom tyrosinase usually catalyzes the conversion of *o*-diphenols to *o*-benzoquinone. However, while catalyzing the oxidation of 3,4-dihydroxymandelate, **39,** this enzyme performs an unusual oxidative decarboxylation reaction to yield 3,4-dihydroxybenzaldehyde, **20,** as the sole product (*43*). During this reaction, neither 3,4-dihydroxybenzyl alcohol, **40,** nor 3,4-dihydroxyphenylglyoxylic acid, **17,** seems to be generated as the intermediate. A simultaneous oxida-



SCHEME 7

tive decarboxylation of substrate to a quinone methide derivative, **41**, and tautomerization of the unstable **41** to the more stable **20** were proposed to account for this novel reaction (Scheme 7). Apart from mushroom tyrosinase, a number of other phenoloxidases, including cuticular phenoloxidase, catalyze this reaction (*43*). It appears from these results that if a suitable substrate such as **39** is provided to phenoloxidases, which are known to catalyze the conversion of catechols to quinones, they will catalyze the quinone methide production as well. Both quinone formation and quinone methide production from 4-alkyl-substituted catechols involve a two-electron oxidation (Scheme 8). Thus *N*-acetyldopamine quinone methide, **42**, is a tautomer of **2.** Therefore, with appropriate alternations at the active site, the same enzyme could catalyze the formation of both products. Alternately, different phenoloxidases can catalyze either quinone formation or quinone methide production from 4-alkylcatechol. Since phenoloxidases from various organisms are known to catalyze the conversion of catechols to the corresponding quinones, quinone formation in cuticle was accepted without detailed experimentation, and the possibility of quinone methide production has never been explored.



SCHEME 8

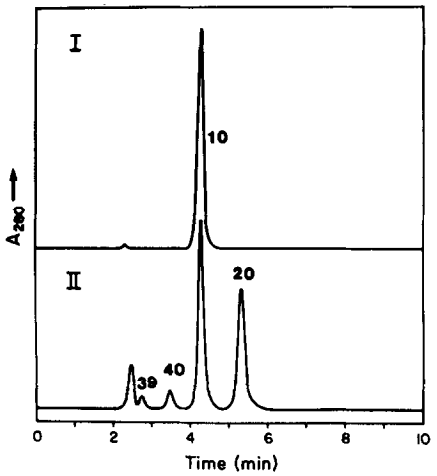
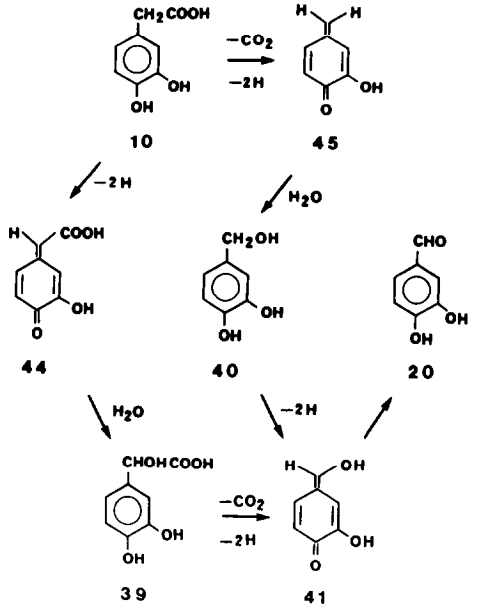


FIG. 2. HPLC of products of 3,4-dihydroxyphenylacetic acid oxidation by cuticular phenoloxidase from *Sarcophaga bullata*. A reaction mixture containing 1 μmol of 3,4-dihydroxyphenylacetic acid, **10**, and 50 mg of cuticular phenoloxidase in 2 ml 0.05 M sodium phosphate buffer, pH 6.0, was incubated at room temperature. At zero time (10 μl, trace I) and after 2 h of incubation (20 μl, trace II), supernatant from the reaction mixture was subjected to HPLC as outlined by Sugumaran and Lipke (*39*). Products identified are 3,4-dihydroxymandelic acid, **39**, 3,4-dihydroxybenzyl alcohol, **40**, and 3,4-dihydroxybenzaldehyde, **20**.

To explore this possibility, we examined the oxidation products of a number of catechols by cuticular phenoloxidase from *Sarcophaga bullata* (*38, 39*). The chitin-bound phenoloxidase from this organism readily oxidized a number of catecholic compounds such as catechol, 3-methylcatechol, 4-methylcatechol, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylglycol, 3,4-dihydroxyphenethyl alcohol, caffeic acid, 3,4-dihydroxy-phenylpropionic acid, chlorogenic acid, dopa, dopamine, *N*-acetyldopamine, *N*-β-alanyldopamine, epinephrine, and norepinephrine. During the oxidation of certain catechols, especially 3,4-dihydroxyphenethyl alcohol, *N*-acetyldopamine, and *N*-β-alanyldopamine, quinone formation could not be visualized, as evidenced by the lack of generation of quinone-type spectra in the visible region. The bulk of the catechol oxidized was bound to the chitin–protein assembly covalently. However, a small portion of it remained soluble in the reaction mixture. Analysis of the reaction mixture by HPLC revealed the generation of side chain oxidized compounds. For instance, Fig. 2 illustrates the HPLC of a reaction mixture containing 3,4-dihydroxyphenylacetic acid and cuticle. Before the reaction is started, the only catecholic compound observed on HPLC is **10** (trace I). After 2 h of incubation with cuticular phenoloxidase, generation of 3,4-dihydroxymandelic acid, **39**, 3,4-dihydroxybenzyl alcohol, **40**, and 3,4-dihydroxybenzaldehyde, **20**, could be witnessed in the reaction mixture (trace II). The reaction course can be explained by the intermediary formation of quinone methides rather than quinones as the initial product of oxidation, as shown in Scheme 9. Similarly, 4-methylcatechol, **46**, was oxidized to 3,4-dihydroxybenzyl



SCHEME 9

alcohol, **40,** and 3,4-dihydroxybenzaldehyde, **20,** probably through the quinone methides **45** and **41.** It is important to recall at this point that both 4-methylcatechol, **46,** and 3,4-dihydroxyphenylacetic acid, **10,** cannot form dehydro derivatives. Apart from 4-methylcatechol and 3,4-dihydroxyphenylacetic acid, a number of other 4-substituted catechols such as 3,4-dihydroxyphenethyl alcohol, *N*-acetyldopamine, and *N*-β-alanyldopamine also exhibited similar reactivities (Table 3). In this context, it is interesting to note that the conversion of *N*-acetyldopamine to *N*-acetylnorepinephrine and of *N*-β-alanyldopamine to *N*-β-alanylnorepinephrine has also been observed in *Manduca sexta* cuticle (*16, 45*).

As stated earlier, the bulk of these catechols was bound to cuticle with retention of *o*-dihydroxyphenolic OH groups. Upon acid hydrolysis, such cuticle released large amounts of the bound catechols. HPLC analysis of the solubilized catechols revealed the presence of side chain modified catechols, confirming the involvement of side chains in adduct formation (Table 3).

Based on these findings, the course of β-sclerotization can be depicted as shown in Scheme 10. The sclerotizing precursor **1** is oxidized by cuticular phenoloxidase to its corresponding quinone methide derivatives, which accounts for the unique release of tritium from the β-position of tritiated *N*-acetyldopamine observed during the cuticular enzyme-catalyzed reaction (*40, 41*).

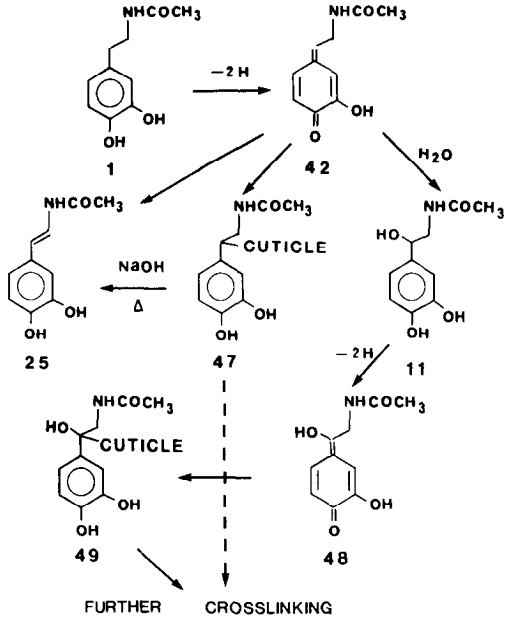
Quinone methides can be formed either directly from substituted catechols by a two-electron oxidation or through corresponding semiquinones by two consecutive one-electron oxidations (Scheme 8). Irrespective of the mode of generation, their formation in cuticle can account for several conflicting observations. Quinone methides are relatively short-lived, highly reactive intermediates (*46*).

TABLE 3

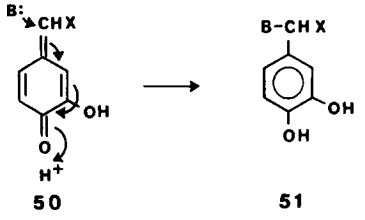
PRODUCT ANALYSIS OF CUTICULAR PHENOLOXIDASE REACTION

Substrate used	Soluble catecholic product formed	Catecholic product released from cuticle
Catechol	None	None
3,4-Dihydroxybenzoic acid	None	None
4-Methylcatechol	3,4-Dihydroxybenzyl alcohol and 3,4-Dihydroxybenzaldehyde	3,4-Dihydroxybenzaldehyde
3,4-Dihydroxyphenylacetic acid	3,4-Dihydroxymandelic acid and 3,4-dihydroxybenzaldehyde	3,4-Dihydroxybenzaldehyde
3,4-Dihydroxyphenethyl alcohol	3,4-Dihydroxyphenyl glycol and 2-hydroxy-3',4'-dihydroxyacetophenone	3,4-Dihydroxybenzaldehyde
N-Acetyldopamine	*N*-Acetylnorepinephrine	(*N*-acetyl)norepinephrine
N-β-Alanyldopamine	*N*-β-Alanylnorepinephrine	(*N*-β-Alanyl)norepinephrine

Note. Larval cuticle (500 mg) from *Sarcophaga bullata*, prepared as outlined in an earlier publication (*39*), was suspended in 50 ml 0.05 M sodium phosphate buffer (pH 6.0) with 100 μmol of specified catechol at 37°C for 1 h. The reaction was arrested by the addition of 0.5 ml concentrated HCl and filtered. Ether-soluble phenols were extracted from the filtrate. Aqueous layers were lyophilized, taken up in methanol, filtered, and used for HPLC analysis. Residual cuticle was washed successively with 200 ml water, 100 ml 2% sodium borate–1% SDS, 500 ml water, 50 ml 1 M HCl, 200 ml water, and 50 ml acetone and dried in air. Hydrolysis of *o*-diphenol-treated cuticle was performed at 108°C for 24 h with 1 M HCl. HPLC analysis of catechols was performed as described in (*39*).



SCHEME 10



50 51

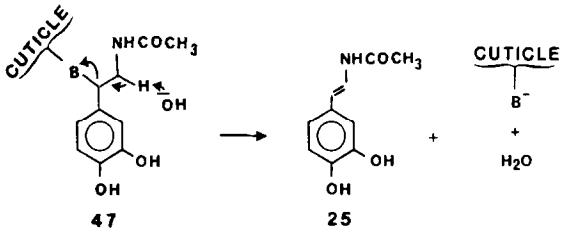
SCHEME 11

They react rapidly with any nucleophiles in the vicinity and form 1,6-addition products with the regeneration of the benzenoid ring (Scheme 11). These reactions convert their unstable quinonoid ring structure to a stable benzenoid structure. Therefore, they are highly favored over other reactions. It is this property that makes the quinone methides the attractive sclerotizing agents for β-sclerotization

Although in nonaqueous solution quinine methides could exist as free intermediates, in aqueous systems they rapidly undergo hydration to yield side chain hydroxylated compounds. Nonenzymatic water additions to **42** would be nonstereospecific and produce equal amounts of *d* and *l* isomers of *N*-acetylnorepinephrine, **11.** This is observed to be so in reality, supporting the participation of quinone methides in β-sclerotization (*42*).

In addition, quinone methide, **42,** can add on to a number of nucleophiles on the protein side chains as well as chitin, forming cuticle–catechol adducts, **47.** The available nucleophiles include the ε-amino group of lysyl residues, the imidazoyl group of histidine residues, the hydroxyl groups of chitin backbone and serine and threonine residues, the carboxyl groups of aspartic and glutamic acids, the thiol group of cysteine, the thioether group of methionine, N-terminal and C-terminal residues of proteins, and free amino groups of chitin.

Quinone methide–cuticle adducts, **47,** will not have any absorption maximum in the visible region in accordance with the property of related catechols. Therefore they will acknowledge the formation of sclerotized but colorless cuticle. These adducts, **47,** upon acid hydrolysis, will provide group II catechols listed in Table 1. The excess *N*-acetylnorepinephrine, **11,** formed can also be converted to its quinone methide derivative, **48,** by the nonspecific cuticular phenoloxidase and used for adduct formation. These adducts, **49,** would produce group II catechols in Table 1 upon acid hydrolysis. Further oxidation of cuticle-bound catechols and coupling to other structural units provide crosslinks necessary for the stabilization of the cuticle. Moreover, quinone methide participation in β-sclerotization also accounts for the generation of 1,2-dehydro-*N*-acetyldopamine, **25,** from cuticle. Since hot alkali treatment was used to isolate this compound (*34*), the double bond seems to have been introduced by a β-elimination reaction. Cuticular quinone methide adduct, **47,** can readily yield **25** upon alkali treatment as shown in Scheme 12. Thus, **25** seems to be an artifically generated compound. Our trapping experiments with sarcophagid cuticular phenoloxidase did not reveal the formation of this compound as an intermediate during the oxidation of *N*-acetyldopamine (*44*). Even if this compound is formed *in vivo* in other organisms, it would be the result

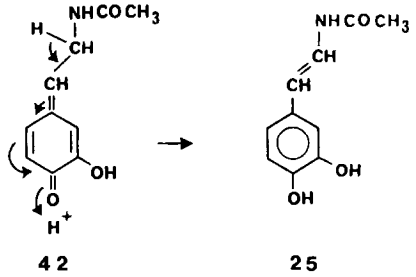


SCHEME 12

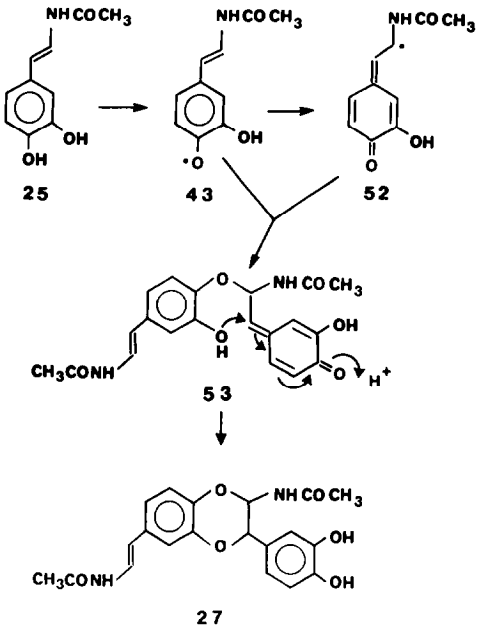
of a prototropic transformation of quinone methide, rather than the action of side chain desaturase (Scheme 13). Such a contention will also be in agreement with Andersen's finding that the β-activating activity (putative desaturase) and the phenoloxidase activity may be due to the same enzyme (*47*).

The relationship of the dimers isolated by Andersen to β-sclerotization is not clear at present. Since these dimers are isolated in extremely small amounts (0.02% by weight of cuticle; in contrast, catechols are present in as much as 14%), their role in β-sclerotization seems to be unimportant. However, their biosynthesis is likely to occur through an oxidative coupling of dehydro compound **25** via quinone methide derivative **52,** as shown in Scheme 14, without the intermediary formation of quinone. Reaction of **52** with other catechol radicals arising from **1, 11, 13, 15, 20,** and **9** would produce the dimers **22, 28, 29, 30, 31,** and **32,** respectively. Thus, quinone methide participation in β-sclerotization accounts for all the observations so far made on the cuticle.

Analogous to quinone tanning where quinones are generated and used up for crosslinking structural components, during β-sclerotization tautomeric quinone methides are produced and consumed for crosslinking. Therefore, we wish to rename β-sclerotization as quinone methide sclerotization. Apart from *N*-acetyldopamine, other 4-alkyl-substituted catechols, such as *N*-acetylnorepinephrine, *N*-β-alanyldopamine, *N*-β-alanylnorepinephrine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenethyl alcohol, and 3,4-dihydroxybenzyl alcohol, can also participate in quinone methide sclerotization. Thus it seems to be a general scheme applicable for a variety of compounds.



SCHEME 13



SCHEME 14

Quinone methides have also been implicated as reactive intermediates during the biosynthesis of lignins (*48*) and tannins (*49*), the enzyme-catalyzed oxidoreductions of anthracycline antitumor antibiotics (*50–52*), and the action of antijuvenile hormones like precocene (*53*). Studies on cuticular phenoloxidase and the reactions of enzymatically generated quinone methides with cuticular components are in progress in this laboratory to unravel the molecular mechanisms of β-tanning in detail.

ACKNOWLEDGMENT

Financial support for this project is provided by USPHS Grant 2ROI-AI-14753.

REFERENCES

1. CHAPMAN, D. F. (1982) The Insects: Structure and Function, pp. 501–528, Harvard Univ. Press, Cambridge.
2. HEPBURN, H. R. (1976) The Insect Integument, pp. 22–496, Elsevier, Amsterdam.
3. KERKUT, G. A., AND GILBERT, L. I. (1985) Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 3, Pergamon Press, Oxford.
4. PRYOR, M. G. M. (1940) *Proc. R. Soc. London Ser. B* **128,** 378–392.
5. PRYOR, M. G. M. (1940) *Proc. R. Soc. London Ser. B* **128,** 393–407.
6. HACKMAN, R. H., AND TODD, A. R. (1953) *Biochem. J.* **55,** 631–637.

7. HACKMAN, R. H. (1974) *in* "The Physiology of Insecta (Rockstein, M., Ed.), Vol. 6, pp. 215–270, Academic Press, New York.
8. PAU, R. (1975) *in* Biology of Arthropod Cuticle (Neville, A. C., Ed.), p. 126, Springer-Verlag, Heidelberg.
9. KARLSON, P., AND SEKERIS, C. E. (1976) *in* The Insect Integument (Hepburn, H. R., Ed.), pp. 145–156. Elsevier, Amsterdam.
10. LIPKE, H., SUGUMARAN, M., AND HENZEL, W. (1983) *Adv. Insect Physiol.* **17,** 1–84.
11. KARLSON, P., AND SEKERIS, C. E. (1962) *Nature (London)* **195,** 183–184.
12. HOPKINS, T. L., MORGAN, T. D., ASO, Y., AND KRAMER, K. J. (1982) *Science* **217,** 364–366.
13. HACKMAN, R. H., AND GOLDBERG, M. (1977) *Insect Biochem.* **7,** 175–184.
14. VINCENT, J. F. V., AND HILLERTON, J. E. (1979) *J. Insect Physiol.* **25,** 653–658.
15. HILLERTON, J. E., AND VINCENT, J. F. V. (1979) *J. Insect Physiol.* **25,** 957–963.
16. PETER, M. G. (1980) *Insect Biochem.* **10,** 221–227.
17. SUGUMARAN, M., HENZEL, W., AND LIPKE, H. (1981) *Fed. Proc.* **40,** 1846.
18. SUGUMARAN, M., AND LIPKE, H. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2480–2484.
19. SUGUMARAN, M., HENZEL, W. J., MULLIGAN, K., AND LIPKE, H. (1982) *Biochemistry* **21,** 6509–6515.
20. SUGUMARAN, M., AND LIPKE, H. (1983) *Insect Biochem.* **13,** 307–312.
21. ANDERSEN, S. O. (1970) *J. Insect Physiol.* **16,** 1951–1959.
22. ANDERSEN, S. O. (1971) *Insect Biochem.* **1,** 157–170.
23. ANDERSEN, S. O. (1974) *Nature (London)* **251,** 507–508.
24. ANDERSEN, S. O., AND BARRETT, F. M. (1971) *J. Insect Physiol.* **17,** 69–83.
25. ANDERSEN, S. O. (1975) *J. Insect Physiol.* **21,** 1225–1232.
26. BARRETT, F. M. (1977) *Insect Biochem.* **7,** 209–214.
27. ANDERSEN, S. O., AND ROEPSTORFF, P. (1978) *Insect Biochem.* **8,** 99–104.
28. ROEPSTORFF, P., AND ANDERSEN, S. O. (1980) *Biomed. Mass Spectrom.* **7,** 317–320.
29. BARRETT, F. M. (1980) *Canad. Entomol.* **112,** 151–157.
30. ANDERSEN, S. O. (1972) *J. Insect Physiol.* **18,** 527–540.
31. ANDERSEN, S. O., JACOBSEN, J. P., AND ROEPSTORFF, P. (1980) *Tetrahedron* **36,** 3249–3252.
32. ANDERSEN, S. O., AND ROEPSTORFF, P. (1981) *Insect Biochem.* **11,** 25–31.
33. ROEPSTORFF, P., AND ANDERSEN, S. O. (1981) *Biomed. Mass Spectrom.* **8,** 174–178.
34. ANDERSEN, S. O., AND ROEPSTORFF, P. (1982) *Insect Biochem.* **12,** 269–276.
35. ANDERSEN, S. O. (1985) *in* Comprehensive Insect Physiology, Biochemistry, and Pharmacology (Kerkut, G. A., and Gilbert, L. I., Eds.), Vol. 3, pp. 59–74, Pergamon Press, Oxford.
36. PIERPOINT, W. S. (1969) *Biochem. J.* **112,** 609–616.
37. JANES, N. F. (1969) *Biochem. J.* **112,** 617.
38. SUGUMARAN, M., AND LIPKE, H. (1983) *Fed. Proc.* **42,** 1828.
39. SUGUMARAN, M., AND LIPKE, H. (1983) *FEBS Lett.* **155,** 65–68.
40. ANDERSEN, S. O. (1976) *in* The Insect Integument (Hepburn, H. R., Ed.), pp. 121–144, Elsevier, Amsterdam.
41. ANDERSEN, S. O. (1977) *Symp. Zool. Soc. London* **39,** 7–32.
42. PETER, M. G. AND VAUPEL, W. (1985) *J. Chem. Soc. Chem. Commun.,* 848–850.
43. SUGUMARAN, M. (1986) *Biochemistry* **25,** 4489–4492.
44. SUGUMARAN, M. (1986) *J. Cell Biochem. Suppl.* **10C,** 92.
45. HOPKINS, T. L., MORGAN, T. D., KRAMER, K. J., ROSELAND, C. R., CZAPLA, T. H., AND ELONEN, R. A. (1986) *J. Cell Biochem. Suppl.* **10C,** 87.
46. WAGNER, H. U., AND GRAOMPPER, R. (1974) *in* The Chemistry of the Quinonoid Compounds (Patai, S., Ed.), pp. 1145–1178, Wiley, New York.
47. ANDERSEN, S. O. (1979) *Insect Biochem.* **9,** 233–239.
48. LEARY, G. J. (1980) *Wood Sci. Technol.* **14,** 21–34.
49. HEMINGWAY, R. W., AND FOO, L. Y. (1983) *J. Chem. Soc. Chem. Commun.,* 1035–1037.
50. MOORE, H. W. (1977) *Science* **197,** 527–532.
51. WALSH, C. (1982) *Tetrahedron* **38,** 871–909.
52. RAMAKRISHNAN, K., AND FISHER, J. (1983) *J. Amer. Chem. Soc.* **105,** 7187–7188.
53. BOWERS, W. S., EVANS, P. H., MARSELLA, P. A., SODERLUND, D. M., AND BETTERINI, F. (1982) *Science* **217,** 647–648.